

Increased and Altered Fragrance of Tobacco Plants after Metabolic Engineering Using Three Monoterpene Synthases from Lemon

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Wild-type tobacco (*Nicotiana tabacum*) plants emit low levels of terpenoids, particularly from the flowers. By genetic modification of tobacco cv Petit Havana SR1 using three different monoterpene synthases from lemon (*Citrus limon* L. Burm. f.) and the subsequent combination of these three into one plant by crossings, we show that it is possible to increase the amount and alter the composition of the blend of monoterpenoids produced in tobacco plants. The transgenic tobacco plant line with the three introduced monoterpene synthases is emitting β -pinene, limonene, and γ -terpinene and a number of side products of the introduced monoterpene synthases, from its leaves and flowers, in addition to the terpenoids emitted by wild-type plants. The results show that there is a sufficiently high level of substrate accessible for the introduced enzymes.

Plants are producing a wide range of different volatile metabolites by different biosynthetic pathways, each having their own complex regulation (Dudareva and Pichersky, 2000). The regulated emission of volatiles from flowers has been suggested to correlate with the patterns of activity of the organisms that are interacting with the plant such as pollinators, herbivores, and predators of the herbivores (Kolossova et al., 2001; Pichersky and Gershenzon, 2002). Emission of volatiles was shown to be regulated at the gene expression level and often displays a nocturnal or diurnal circadian rhythm as was shown for methylbenzoate emission of snapdragon (*Antirrhinum majus*), tobacco, and *Clarkia breweri* (Dudareva et al., 1996, 2000; Kolossova et al., 2001).

Terpenoid synthases are to a large extent responsible for the diversity of terpenoid structures involved in floral scent or in herbivore induced volatiles (Bohlmann et al., 1998). Monoterpenes are C₁₀ compounds, synthesized from geranyl diphosphate (GPP) via the plastid localized 2C-methyl-D-erythritol-4-phosphate (MEP) pathway (Eisenreich et al., 1997). They are of high economic value because they are products extensively used as flavor and fragrance additives in food and cosmetics (Verlet, 1993). Furthermore, they are of interest for their me-

dicinal, e.g. anticarcinogenic, properties (Crowell, 1999). Their biological significance lies in their involvement in plant-insect, plant-pathogen, and plant-plant interactions (Wink, 1999). Monoterpenes can be released in a diurnal rhythm as was shown for rose flowers (Helsper et al., 1998, 2001). A monoterpene synthase mRNA from *Artemisia annua* has recently been shown to be regulated in a circadian rhythm, and the expression profile was shown to correlate with the content and emission of the monoterpene produced by the corresponding enzyme (Lu et al., 2002). Monoterpene emission is also affected by environmental conditions such as temperature and light (Schuh et al., 1997). Tobacco varieties have also been investigated for their emission profiles and were shown to emit mono- and sesquiterpenes. Emission of volatiles from leaves was 30- to 100-fold lower than emission from flowers (Andersen et al., 1988; Loughrin et al., 1990).

Genetic modification offers new opportunities to study the regulation of the biosynthesis of secondary metabolites in plants. Several applications of genetic modification of terpenoid pathways have been reported. In rice (*Oryza sativa*; Ye et al., 2000), the entire vitamin A biosynthetic pathway was created by introducing the genes encoding this pathway. In tomato (*Lycopersicon esculentum*; Romer et al., 2000) and rape (Shewmaker et al., 1999) the carotenoid level was increased by overexpression of phytoene synthase. Using sense and antisense approaches, levels of mono- and diterpenoids could be altered (Mahmoud and Croteau, 2001; Wang et al., 2001). Also, several monoterpene synthases have been introduced into plants, resulting in a change of the aroma profile,

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in the production of new monoterpenes that are emitted from vegetative and floral tissues, or in the accumulation of the corresponding glucoside (Lewinsohn et al., 2001; Lückner et al., 2001; Lavy et al., 2002). However, no reports have been published on the simultaneous introduction of multiple enzymes competing for the same substrate. In this paper, we report on the changes of the volatile profile of tobacco by metabolic engineering, using three different monoterpene synthases from lemon (*Citrus limon*) and the subsequent combination of all three synthases into one plant by crossing.

RESULTS

Genetic Analysis of the Transformed Plant Lines

The young developing transgenic plants, transformed with monoterpene synthase cDNA from lemon, were analyzed for expression of the cDNAs by northern blotting. Figure 1 shows that there are large differences in expression levels of the three monoterpene synthase cDNAs between the independent transgenic lines.

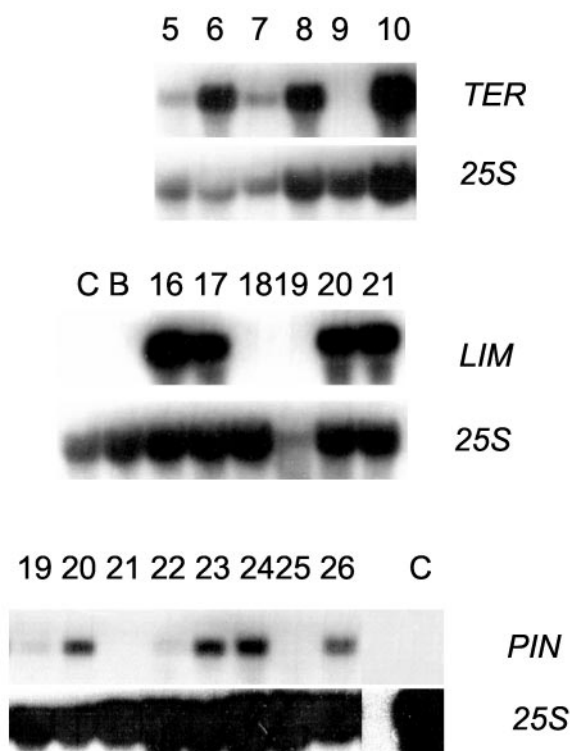


Figure 1. Northern-blot analysis of tobacco plant lines transformed with monoterpene synthases from lemon. Top panels show expression in tobacco leaves of the cDNAs *TER* (γ -terpinene synthase), *LIM* (limonene synthase), and *PIN* (β -pinene synthase) in a subset of the plant lines. The bottom panels show a 25S ribosomal cDNA control to indicate loading differences. C, SR1 non-transgenic control plant; B, empty binary vector transformed line. Numbers indicate transgenic plant lines.

The number of inserts in the transgenic lines was determined by Southern blotting, which showed that some plants contained more than one insert (data not shown). Lines were selected with integration of a cDNA on a single locus to simplify phenotypic and genetic analysis of the transgenic lines. The results of the Southern blotting were verified by seed plating experiments after self-pollination of all the primary transformants and control plants. All germinated seeds of the control plants were sensitive to kanamycin (Table I). As expected, plant lines shown to have multiple gene insertions by Southern blotting (e.g. TER-8, LIM-20, and PIN-24) did not show 1:3 segregation for kanamycin sensitivity (Table I). The plant lines TER-6, PIN-26, and LIM-21, having only one cDNA insertion according to the Southern blot, did show the 1:3 segregation (Table I). The progeny of the cross between TER-6 and PIN-26, each transformed with a different cDNA and both with a single-gene insertion, also showed the expected 1:3 segregation for kanamycin sensitivity (Table I), indicating that the two different cDNAs were integrated on independent loci. Plant lines TER-6, PIN-26, and LIM-21 were selected for further analysis of monoterpene production.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of the Transformed Plants

Terpene production was determined by headspace solid phase micro-extraction (SPME) measurements on CaCl_2 extracts of young leaves of the control plants and several transgenic lines. In control SR1 leaves, no production of monoterpenes could be detected (Fig. 2). Transformed plant lines expressing the different constructs produced the same compounds as were identified by *Escherichia coli* expression of the genes (Lückner et al., 2002). Plant lines expressing the *TER* cDNA showed production of mainly γ -terpinene (5), but also trace amounts of α -terpinene (3) and limonene (4). Plant lines expressing the *LIM* cDNA produced only limonene (4). Plant lines expressing the *PIN* cDNA produced mostly β -pinene (2) and traces of sabinene (1) and γ -terpinene (5).

Combination of Three Monoterpene Synthases in One Plant by Crossing

Crossings were made between primary transformants containing one insert of one of the three monoterpene synthase cDNAs. The first crossing was made between plant lines TER-6 and PIN-26 to combine β -pinene and γ -terpinene synthases. Headspace SPME measurements on intact, 4-week-old F_1 seedlings resulting from this crossing showed emission of both β -pinene and γ -terpinene, emission of single products, or no detectable emission of any of these monoterpenes (data not shown). A plant emitting

Table 1. Seed plating experiments on kanamycin-containing medium for different transgenic lines of tobacco

Seedlings that turn white after germination are kanamycin sensitive (Kan^S). Because all terpene synthase binary constructs also contain a kanamycin resistance (Kan^R) gene on the same T-DNA, the no. of kanamycin resistance T-DNA insertions is supposed to be equal to the no. of monoterpene synthase T-DNA insertions. Both selfings of mother plants (S₁) and crosses between mother plants (F₁) were made, and the progeny of these crosses also showed a Mendelian segregation for the kanamycin resistance gene. For 100 seeds, the critical X² value for 1 degree of freedom and a *P* value of 0.05 is 3.841. If H₀: Kan S: Kan R = 1:3 is true, the transgenic mother plant contains a T-DNA insertion only on a single locus; otherwise, H₁ is true, which implies that more than one insert is present. Plants TER-6, LIM-21, and PIN-26 were selected for further experiments because these plants were containing only one gene insertion based on these seed plating observations. The progeny of seeds of these plants and also of the cross between TER-6 and PIN-26 showed the expected 1:3 ratio for kanamycin sensitivity.

| | Plant Line | Kan ^S | Kan ^R | X ² _{KanS} | X ² _{KanR} | X ² _{total} | |
|----|---------------|------------------|------------------|--------------------------------|--------------------------------|---------------------------------|----|
| | SR-1 | 100 | 0 | | | | |
| S1 | TER-6 | 27 | 89 | 0.1379 | 0.0449 | 0.183 | H0 |
| S1 | TER-10 | 24 | 76 | 0.04 | 0.013 | 0.053 | H0 |
| S1 | TER-8 | 6 | 94 | 14.44 | 4.81 | 19.25 | H1 |
| S1 | LIM-21 | 23 | 86 | 0.6628 | 0.2209 | 0.8837 | H0 |
| S1 | LIM-20 | 2 | 98 | 21.16 | 7.05 | 28.21 | H1 |
| S1 | PIN-26 | 30 | 70 | 1 | 0.33 | 1.33 | H0 |
| S1 | PIN-24 | 6 | 94 | 14.44 | 4.813 | 19.25 | H1 |
| F1 | TER-6×PIN-26 | 28 | 72 | 0.36 | 0.12 | 0.48 | H0 |
| F1 | LIM-21×PIN-24 | 16 | 84 | 3.24 | 1.08 | 4.32 | H1 |

both β -pinene and γ -terpinene at high levels was crossed with the limonene producing LIM-21 primary transformant. Figure 3 shows the results of headspace SPME measurements of the 4-week-old intact seedlings resulting from this second cross. Seven of the

eight possible phenotypes were detected in a population of 20 plants analyzed, shown in Figure 3, A to G. Figure 3G shows the GC-MS profile of a plant emitting all three main products of the introduced monoterpene synthases (TERLIMPIN-plant).

Headspace analysis by Tenax trapping of volatiles released by young leaves of mature TERLIMPIN-plants showed emission of all three main products, β -pinene (4), limonene (7), and γ -terpinene (8; Fig. 4), emitted at levels of 150 to 350 ng g⁻¹ 24 h⁻¹. In every headspace analysis of leaves of the TERLIMPIN-plant line, limonene was more abundant than the main products of the other two monoterpene synthases. The leaves of the control plant emitted trace amounts of the monoterpenoids α -pinene (2), β -pinene (4), and linalool (10; Fig. 4A) at levels of 5 to 15 ng g⁻¹ 24 h⁻¹. In the headspace of the transgenic TERLIMPIN-plant, also trace amounts were identified of α -thujene (1), myrcene (5), terpinolene (9), and linalool (10) at a level of 5 to 15 ng g⁻¹ 24 h⁻¹, whereas α -pinene (2), sabinene (3), and *p*-cymene (6) were emitted at a slightly higher level of 20 to 50 ng g⁻¹ 24 h⁻¹ (Fig. 4B). In addition, the sesquiterpene β -caryophyllene was emitted by the leaves of both the control and the transgenic plant (66 and 42 ng g⁻¹ 24 h⁻¹, respectively; data not shown). Total amounts of monoterpenes emitted from young transgenic leaves were approximately 5- to 10-fold higher than those emitted from older transgenic leaves (data not shown).

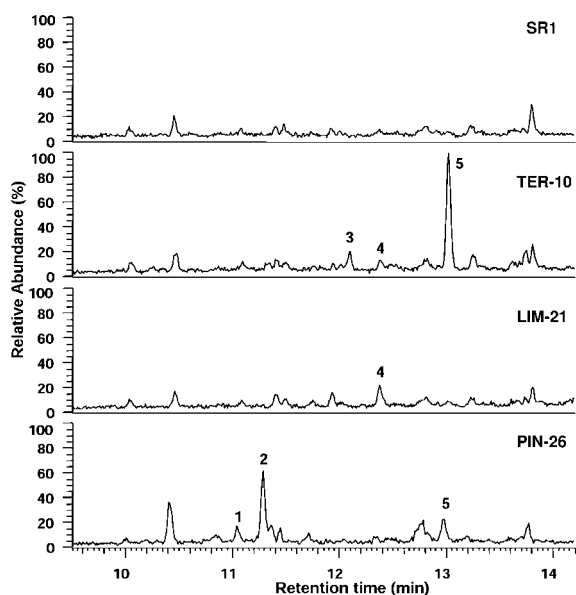


Figure 2. Typical GC-MS chromatograms showing the volatiles of leaves of a wild-type tobacco control plant and transformant lines expressing three monoterpene synthases from lemon. The chromatograms were obtained using headspace SPME of the ground leaf material in 2.5 M CaCl₂. SR1, Non-transformed control plant; TER-10, transgenic plant line expressing γ -terpinene synthase. LIM-21, Transgenic plant line expressing limonene synthase. PIN-26, Transgenic plant line expressing β -pinene synthase. y axis is showing relative abundance of m/z 67 + 68 + 69 + 79 + 93 + 121 + 136 with 100% level set at 2.01 × e⁴. All peaks that represent monoterpenes are numbered; they were identified by comparing full mass spectra and retention times to authentic standards. 1, Sabinene; 2, β -pinene; 3, α -terpinene; 4, limonene; 5, γ -terpinene.

Monoterpenes Emitted during Flower Development

Volatiles emitted from different flowering stages were collected for 24 h using Tenax trapping and analyzed by GC-MS. Control flowers emitted linalool (9) and β -caryophyllene (10; Fig. 5, A–D). In the headspace of transgenic flowers, all of the main prod-

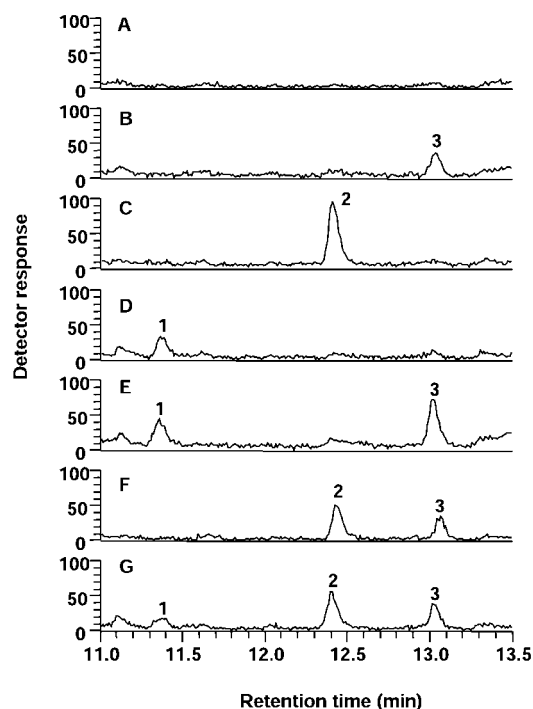


Figure 3. GC-MS headspace chromatograms measured using headspace SPME of 4-week-old seedlings showing different progeny phenotypes from a crossing between an F_1 hybrid of TER-6 and PIN-26 (emitting both β -pinene and γ -terpinene) and LIM-21, a primary transgenic line emitting limonene (A–G). The GC-MS trace shows the combination of m/z 67 + 68 + 69 + 71 + 79 + 91 + 93 + 105 + 107 + 121 + 136. 1, β -pinene; 2, limonene; 3, γ -terpinene. A, The profile of a plant that lost all three terpene synthase activities, because there are no monoterpenes emitted anymore. B through D, Plants that emit only γ -terpinene, limonene, or β -pinene, respectively. E and F, Plants that emit two main products of the introduced monoterpene synthases β -pinene and γ -terpinene (E) or limonene and γ -terpinene (F). G, A plant that emits all the three main products of the introduced monoterpene synthases.

ucts of the three introduced lemon monoterpene synthases, β -pinene (4), limonene (7), and γ -terpinene (8), were detected in all stages (Fig. 5, E–H), in addition to the native linalool (9) and β -caryophyllene (10). Furthermore, trace amounts were detected of α -thujene (1), α -pinene (2), sabinene (3), myrcene (5), and p -cymene (6). The levels of limonene and γ -terpinene emitted decreased during flower development of the transgenic flowers, whereas the level of β -pinene was relatively stable and decreased only in the open flowers. The levels of linalool emitted from control and transgenic flowers were approximately similar. However, the pattern of emission was different when compared with the monoterpenes produced as a consequence of the constitutively expressed monoterpene synthases, because the level of linalool first increased during flower development and then decreased in the open flower (Fig. 5; Table II). For control flowers, the emission of β -caryophyllene steadily increased with flower development, whereas for transgenic flowers, the level

of emission remained relatively stable during the different stages measured. During the later developmental stages, the level of β -caryophyllene emitted by transgenic flowers was about 2- to 3-fold lower than that of control flowers (Table II).

Configuration of Produced Compounds

The enantiomeric composition of the native linalool produced by the control tobacco flowers had an enantiomeric excess (e.e.) of 52% *R*-linalool, identical to the ratio in the transgenic plants. The limonene produced in the limonene synthase plant LIM-21 has an e.e. of almost 100% of (+)-limonene, just like the enantiomeric composition of the limonene produced when this enzyme was expressed in *E. coli*. The limonene produced by flowers of several mixed developmental stages of the plant containing all three monoterpene synthases had an e.e. of 87% (+)-limonene (data not shown). Also the enantiomeric composition of the limonene and β -pinene in the separate flower stages was determined for the Tenax trapped headspace of the flowers of stage 8 (from Fig. 5E) and the flowers of stage 10 (from Fig. 5F). Whereas the flowers of stage 8 that had a high level of limonene had an e.e. of 92% for (+)-limonene, the flowers of stage 10 only had an e.e. of 51% for (+)-limonene. For β -pinene, the e.e. for (–) β -pinene in the flowers of stage 8 was 86%, whereas in the flowers of stage 10, the e.e. for (–) β -pinene was higher, namely 92% (data not shown).

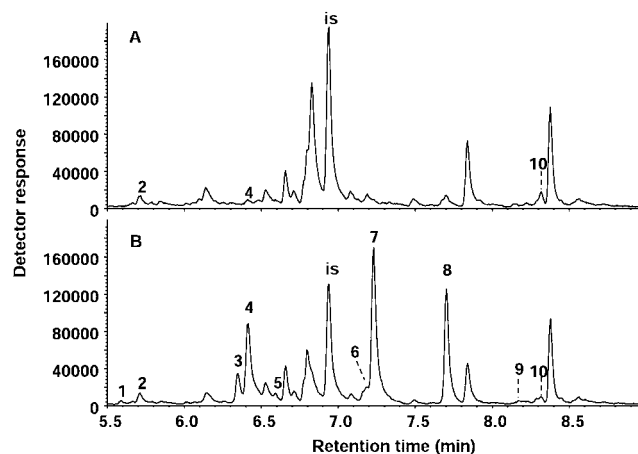


Figure 4. Volatile emission profile of detached leaves of tobacco plants sampled for 24 h using Tenax trapping of a nontransformed control plant (A) and a plant expressing γ -terpinene, (–) β -pinene, and (+)-limonene synthase (B). Monoterpene peaks identified by mass spectrum and retention time are numbered. 1, α -Thujene; 2, α -pinene; 3, sabinene; 4, β -pinene; 5, myrcene; 6, p -cymene; 7, limonene; 8, γ -terpinene; 9, terpinolene; 10, linalool. is, Internal standard (+)-3-carene (212 ng). Non-monoterpene peaks are not labeled.

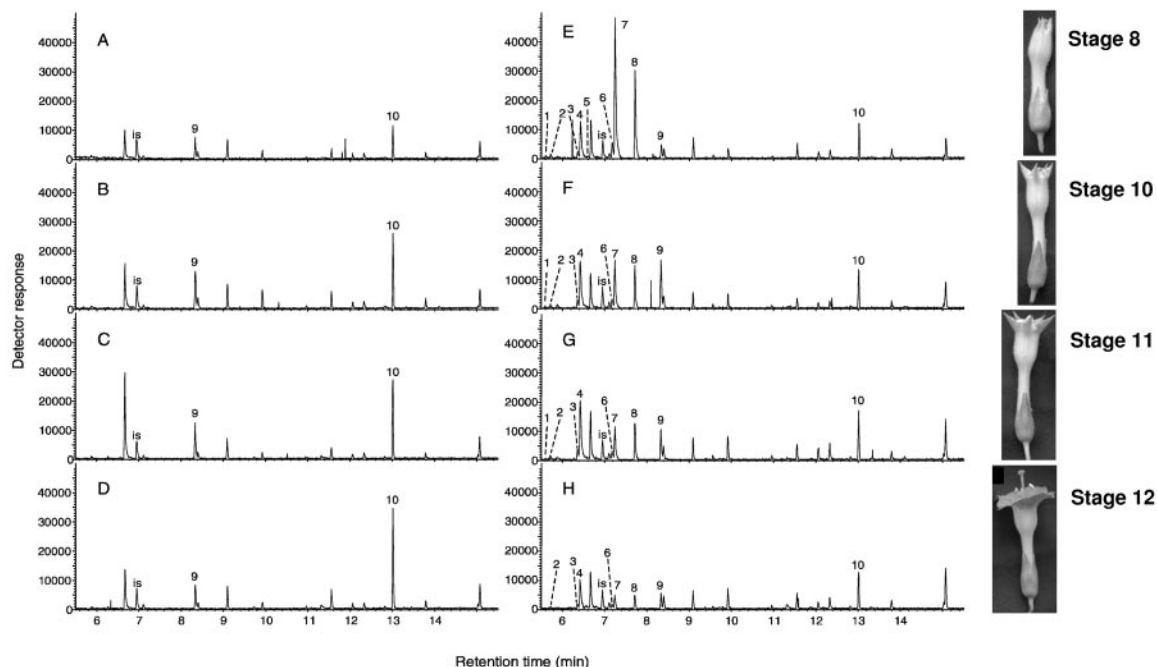


Figure 5. Volatile emission profiles of different developmental stages of detached tobacco flowers sampled for 24 h using Tenax trapping of control (A–D) and transgenic plants with the three lemon monoterpene synthases (E–H; respectively γ -terpinene, (–)- β -pinene, and (+)-limonene synthase). Each time five flowers were sampled. The different stages of the flowers, stages 8, 10, 11, and 12, are depicted next to the chromatograms. Monoterpene and sesquiterpene peaks identified by their mass spectrum and retention times are numbered. 1, 97 -Thujene; 2, β -pinene; 3, sabinene; 4, β -pinene; 5, myrcene; 6, *p*-cymene; 7, limonene; 8, γ -terpinene; 9, linalool; 10, β -caryophyllene. is, Internal standard (+)-3-carene (212 ng). Non-terpene peaks are not labeled.

DISCUSSION

Monoterpenoid Profile

This is the first report on the simultaneous expression of multiple introduced terpene synthases in plants. A large variety of different monoterpene skeletons was introduced into wild-type tobacco plants by metabolic engineering. The total level of the monoterpenoids produced was greatly increased (10- to 25-fold; Fig. 4) and resulted in a drastic change of the fragrance profile emitted from tobacco flowers and leaves.

The altered monoterpene emission profile could be readily detected by the human nose (El Tamer et al., 2003). Therefore, an approach like this could be applied to improve the fragrance of commercially important cut flowers such as roses (Vainstein et al., 2001). A first attempt to achieve this goal with a linalool synthase in carnation did not lead to the emission of large quantities of linalool, probably as a consequence of insufficient availability of the substrate GPP (Lavy et al., 2002). Another reason for a low level of volatile terpenoid production after genetic modification with a monoterpene synthase was found with petunia (*Petunia hybrida*) plants, transformed with linalool synthase. Biochemical analysis revealed that all of the synthesized *S*-linalool was converted into its corresponding non-volatile Glc

conjugate (Lücker et al., 2001). Glycosylation of linalool was not detected in transgenic carnation (Lavy et al., 2002). In the present case, no indications were found for glycosylation of the introduced monoterpenes. However, some further modification did seem to occur. In the transgenic tobacco plants, the levels of *p*-cymene were higher than the levels of α -thujene, α -pinene, α -terpinene, and terpinolene (Figs. 4 and 5). These products were previously reported to be more predominant side products of γ -terpinene synthase than *p*-cymene was during expression in *E. coli* (Lücker et al., 2002). This may be caused by further conversion of monoterpenes by native enzymes of tobacco or by nonenzymatic oxidation reactions induced by light and air. In an early study on the biosynthesis of aromatic monoterpenes in *Thymus vulgaris*, it was suggested that *p*-cymene could be formed from γ -terpinene by the action of a putative desaturase enzyme (Poulose and Croteau, 1978). In cold-pressed *Citrus junus* oil, *p*-cymene concentrations increased upon storage at the expense of e.g. limonene and γ -terpinene (Njoroge et al., 1996). In lemon oil, several monoterpenes decreased while *p*-cymene increased in concentration upon UV irradiation (Iwanami et al., 1997). Other modifications like hydroxylations described for tomato and carnation transformed with linalool synthase (Lewinsohn et al., 2001; Lavy et al., 2002) were not detected in

Table II. Emission patterns of terpenoids from different developmental stages of control and transgenic tobacco flowers

For each stage and plant type, five flowers were detached and placed on floral foam. Control, Non-transgenic SR1 plant; transgenic, TERLIMPIN-plant expressing γ -terpinene synthase, limonene synthase, and β -pinene synthase.

| | Stage 8 | | Stage 10 | | Stage 11 | | Stage 12 | |
|------------------------|--------------------------------------|------------|----------|------------|----------|------------|----------|------------|
| | Control | Transgenic | Control | Transgenic | Control | Transgenic | Control | Transgenic |
| | $\text{ng g}^{-1} 24 \text{ h}^{-1}$ | | | | | | | |
| α -Thujene | 0 | 29 | 0 | 13 | 0 | 14 | 0 | 0 |
| α -Pinene | 0 | 30 | 0 | 31 | 0 | 25 | 0 | 9 |
| Sabinene | 0 | 49 | 0 | 47 | 0 | 55 | 0 | 28 |
| β -Pinene | 0 | 356 | 0 | 371 | 0 | 378 | 0 | 179 |
| ρ -Cymene | 0 | 115 | 0 | 57 | 0 | 48 | 0 | 28 |
| Limonene | 0 | 1206 | 0 | 321 | 0 | 190 | 0 | 71 |
| γ -Terpinene | 0 | 630 | 0 | 237 | 0 | 184 | 0 | 73 |
| Linalool | 127 | 88 | 188 | 254 | 207 | 139 | 121 | 79 |
| β -Caryophyllene | 182 | 190 | 328 | 171 | 420 | 175 | 468 | 151 |

these transgenic tobacco plants. Flowers of the transgenic tobacco plants that were only expressing (+)-limonene synthase produced almost exclusively (+)-limonene, whereas the plant that in addition to the (+)-limonene synthase also expressed the (–)- β -pinene and the γ -terpinene synthases produced a lower e.e. of (+)-limonene. This is probably the result of the additional (–)-limonene produced as side products by the (–)- β -pinene synthase and the γ -terpinene synthase. Around 3% of the total products of (–)- β -pinene synthase and around 7% of the total products of the γ -terpinene synthase, when expressed in *E. coli*, were (–)-limonene (Lücker et al., 2002). Because the levels of the main products of the three monoterpene synthases varied in the different transgenic flower development stages, the variation in e.e. of (+)-limonene is most likely related to the activity of the three different enzymes. Although less obvious, the same conclusion can be drawn for the enantiomeric composition of β -pinene in the flower stages. Table II shows that the level of γ -terpinene in the flowers of stage 8 is higher than the level of β -pinene. The previously reported side product (+)- β -pinene of the γ -terpinene synthase (almost 5% of total products) when expressed in *E. coli* (Lücker et al., 2002) therefore probably contributed to the lower e.e. of 86% (–)- β -pinene in this flower stage compared with the 92% e.e. (–)- β -pinene in the flower stage 10 where the γ -terpinene level is lower and the β -pinene level is only slightly increased.

Precursor Availability and Production Level

Monoterpene formation, which takes place in the plastids, depends on the availability of the substrate GPP, which is produced in the plastids by the MEP pathway (Eisenreich et al., 1997). This in turn is depending on the expression of GPP synthase and on the expression and regulation of genes earlier in the MEP pathway. In all analyzed flower developmental stages, except the first, the level of β -caryophyllene in the transgenic plants was 2- to 3-fold lower than in

control plants. This might be an indication that transport of precursors such as IPP, from the site of production in the plastids to the cytosol, contributes to β -caryophyllene formation in control plants. Such an exchange of precursors from the plastids to the cytosol has been demonstrated for the biosynthesis of other sesquiterpenes such as bisabololoxide A and chamazulene in chamomile (Adam and Zapp, 1998) and for germacrene D in *Solidago canadensis* (Stelio-poulos et al., 2002). A study on *Mentha × piperita* glandular trichomes, using several ^{14}C -radiolabeled substrates showed that sesquiterpenes are exclusively derived from a plastidic pool of precursors (McCaskill and Croteau, 1995, 1998). This supposed transport to the cytosol might be decreased in our transgenic tobacco plants because most of the isopentenyl diphosphate/GPP is used by the introduced monoterpene synthases, which are active at the site of biosynthesis of this precursor and are therefore a better competitor for the GPP than the sesquiterpene synthase residing in the cytosol.

Possibly, the introduction of monoterpene synthases in a transgenic plant also leads to a change in the flux of carbon to GPP, which may imply that there is less precursor available for geranylgeranyl diphosphate (GGPP) and subsequent diterpene and carotenoid formation. Nevertheless, because of the considerable level of emitted terpenoids in tobacco leaves and flowers, it seems likely that a sufficient amount of free substrate is available or can be made available, particularly in young leaves and flower buds. This pool of GPP in young tissues may benefit the plant by allowing the rapid production of monoterpenes to repel herbivores or attract predators of the herbivores and thus protect new growth. Whether the GPP precursors are already available in the plants or whether the biosynthesis is up-regulated upon expression of the introduced monoterpene synthases remains unknown.

At present, different research groups have attempted transformation experiments regarding overexpression of monoterpene or sesquiterpene synthases in different plant species. In our tobacco

monoterpene synthase transformation experiments, the total level of additional monoterpenes emitted in young leaves reached up to 750 ng g⁻¹ fresh weight 24 h⁻¹. In flowers, the levels ranged from 400 to 2,400 ng g⁻¹ fresh weight 24 h⁻¹. In other experiments using *S*-linalool synthase, transgenic petunia accumulated between 1,000 and 10,000 ng g⁻¹ fresh weight of the corresponding *S*-linalyl- β -D-glucopyranoside in leaves (Lücker et al., 2001). With the same gene, transgenic tomato accumulated between 165 and 833 ng g⁻¹ fresh weight *S*-linalool and in addition 199 to 504 ng g⁻¹ fresh weight 8-hydroxylinalool in the fruit (Lewinsohn et al., 2001). Transgenic carnation accumulated approximately 400 ng g⁻¹ dry weight trans-linalool oxide within mature petals, in addition to some non-quantified emission of linalool and cis- and trans-linalool oxide (Lavy et al., 2002). An interesting observation was that in carnation, GPP synthase activity was detected in control plants that do not produce monoterpenes (Lavy et al., 2002), suggesting the presence of a pool of unused GPP. A putative GPP pool available for monoterpene biosynthesis in non-monoterpene-producing plants was also suggested in a recent review on terpenes (Chappell, 2002). Alternatively GPP might be "leaking" from a GGPP synthase during GGPP formation, the precursor of e.g. carotenoids (Lewinsohn et al., 2001).

The levels of product formation after introduction of a monoterpene synthase are much higher than the levels produced after the introduction of a sesquiterpene synthase. When a fungal trichodiene synthase, a sesquiterpene synthase, was transformed to tobacco plants, this resulted in low levels of trichodiene of about 5 to 10 ng g⁻¹ leaf tissue, although there was active trichodiene synthase enzyme present (Hohn and Ohlrogge, 1991). When another sesquiterpene synthase, amorpho-4,11-diene synthase from *A. annua*, was transformed to tobacco, this resulted in an even lower level of 0.2 to 1.7 ng product g⁻¹ fresh weight (Wallaart et al., 2001). Because the accumulated levels are low, there is apparently a low amount of farnesyl diphosphate available in these plants.

Despite the fact that the K_m value of the limonene synthase is 4.5-fold lower than the K_m of the β -pinene and the γ -terpinene synthase (Lücker et al., 2002), the emission levels from flowers of the main products of the introduced monoterpene synthases are comparable. Due to the lower K_m of limonene synthase, in the presence of low concentrations of GPP, the level of limonene should be much higher than that of β -pinene and γ -terpinene. Hence, we assume that in flowers, the GPP pool is large and is not limiting terpene production. This is further supported by the fact that linalool emission is hardly affected by the engineered enzymes competing for the same substrate. In leaves, the levels of compounds produced by the introduced monoterpene synthases are indeed more similar to what would be expected from differ-

ences in K_m between the enzymes. As can be seen in Figure 4, the limonene level is higher than those of the other main products indicating that the level of GPP might be a limiting factor for the production in this tissue.

The amounts of engineered terpenes trapped from the various floral developmental stages show varying degrees of correlation: Limonene and γ -terpinene correlate to r^2 of 0.98, whereas β -pinene emission shows a low correlation with the emission of γ -terpinene (r^2 of 0.24). All engineered monoterpene synthases are driven by the same promoter and thus should exhibit the same expression and, in theory, also the same product formation. We have no explanation for this discrepancy, except perhaps if we assume that β -pinene may be converted to another, unidentified and undetected product by (an) endogenous enzyme(s). The emission patterns of native linalool and β -caryophyllene are quite different from the patterns of the introduced monoterpenes: There is no correlation between the levels of linalool and the levels of the engineered monoterpene (r^2 values of 0.03, 0.06, and 0.3 between linalool and γ -terpinene, limonene, and β -pinene respectively). This is not unexpected, because the native terpenoid synthases are under an endogenous regulation mechanism that will obviously give rise to a gene expression pattern that differs from that of the constitutive cauliflower mosaic virus (CaMV) d35S-induced gene expression. Obviously, regulation of fragrance emission does involve more than gene expression alone. In snapdragon, the gene expression profile of terpene synthase genes showed a similar pattern as the release of terpenes during a day. However, even in that case, the regulation of terpene emission by translational or posttranslational regulation or modification of monoterpenes could not be ruled out (Dudareva et al., 2003).

CONCLUSIONS

The monoterpenes emitted from the transgenic tobacco plants are volatiles that occur in flowers of many plant species (Knudsen et al., 1993) and could therefore attract insect species other than those normally attracted to tobacco. Transgenic plants with altered floral scents have been suggested previously to be ideal models for pollinator behavior studies or to study plant-herbivore interactions (Dudareva and Pichersky, 2000; Pichersky and Gershenzon, 2002). Also, changes in tritrophic interactions may be expected such as in predator and parasite attraction (Dicke and van Loon, 2000). The results presented here show the feasibility to generate plant lines making a variety of new volatiles or change the enantiomeric composition of the volatiles. These results will pave the way for a new approach to chemical ecology and a multitude of future studies using transgenic plants for fascinating ecological studies.

MATERIALS AND METHODS

Plant Material

Tobacco (*Nicotiana tabacum* cv Petite Havana SR1; Maliga et al., 1973) was used as starting material for all transformations. Plants were grown under a 16-h photoperiod and standard greenhouse conditions.

Cloning of Genes in Plant Expression Vectors

From a random sequencing approach on a pBluescript UNIZAP-XR cDNA library (Stratagene Europe, Amsterdam Zuidooit, The Netherlands), three full-length lemon (*Citrus limon* L. Burm. f.) monoterpene cyclases, γ -terpinene cyclase, *TER* (GenBank <http://www.ncbi.nlm.nih.gov/index.html>; accession number, *ClgTERPS*, AF514286), (+)-limonene cyclase 1, *LIM* (*Cl*(+) *LIMS1*: AF514287), and (–)- β -pinene cyclase, *PIN* (*Cl*(–) *BPINS*: AF514288), including a predicted plastid targeting signal, were selected to be transformed to tobacco plants (Lücker et al., 2002). They were subcloned without the pBluescript multiple cloning sites and without the poly(A) tail into the kanamycin resistance gene containing pBinPLUS vector (van Engelen et al., 1995). Genes were cloned by Quickchange PCR according to the manufacturer's recommendations (Stratagene) followed by resequencing of the mutated vector. The PCR program used was 30 s, 95°C; 1 min, 55°C; 12 min, 68°C; 14 cycles on a Mastercycler gradient PCR apparatus (Eppendorf Scientific, Westbury, NY).

For cloning of *TER*, first a *SpeI* site was introduced in the 5'-untranslated region (UTR) using the primers TERF 5'-ggAATTCggCACTAgTATA-gAATCCTTg-3' and its exact complement. For the primers used, the altered nucleotides are written in *italics*, and the introduced restriction site is underlined. After selection by sequencing of the correct clone including the mutation, it was cut with *BglII* and filled in with Klenow fragment (Invitrogen, Breda, The Netherlands) followed by digestion with *SpeI*. The cDNA insert fragment was ligated, using DNA ligase I (Invitrogen) to a pFLAP10 vector fragment (pFLAP10 vector was kindly provided by Dr. A. Bovy, Plant Research International), containing a CaMV d35S promoter and a nopaline synthase terminator sequence that was digested with *SalI*, filled in with Klenow fragment, and subsequently digested with *XbaI*, to obtain a compatible site for *SpeI*. The pFLAP vector now containing the *TER* open reading frame was digested with *PacI* and *AscI* and ligated to the pBinPLUS vector (van Engelen et al., 1995) digested with the same enzymes resulting in plasmid pJLT2.

For cloning of *LIM*, first a *SalI* site was introduced in the 3'-UTR using the primers LIMF 5'-gCagTTTCagTCgACgTTggCCTCCAC-3' and its exact complement. After selection of the correct clone including the mutation, it was cut with *NheI* and *SalI*. The cDNA insert was ligated to a pFLAP10 vector fragment that was digested with *XbaI* and *SalI*. The pFLAP vector was subsequently digested with *PacI* and *AscI* and ligated to pBinPLUS, which was digested with the same restriction enzymes, resulting in a plant expression vector pJLT3.

For cloning of *PIN*, a *BamHI* site was introduced in the 5'-UTR using the primers PINF 5'-ggAATTCggCACgaggaTCCTTgAAATTATATTTC-3' and its exact complement. After selection of the correct clone including the mutation, it was cut with *BamHI* and *AflIII*. The cDNA insert was ligated to a pFLAP10 vector fragment that was digested with *BamHI* and *NcoI*. The resulting vector was digested first with *AscI* and subsequently partially digested with *PacI*, because the 3' end of the open reading frame of the *PIN* cDNA contains a *PacI* site. The fragment including the CaMV d35S promoter and the nopaline synthase terminator was ligated to pBinPLUS digested with *PacI* and *AscI* resulting in the plant expression vector pJLT4. All fragments were isolated from low melting point agarose gel (Sigma-Aldrich Chemie b.v., Zwijndrecht, The Netherlands) using AgarACE agarose digesting enzyme according to the manufacturer's recommendations (Promega Benelux b.v., Leiden, The Netherlands). For subcloning steps, XLBlue MRF⁺ supercompetent cells (Stratagene) were used. Plasmid DNA was isolated from *E. coli* cultures using a plasmid DNA isolation robot (Qiagen GmbH, Hilden, Germany) as described previously (Lücker et al., 2001).

Restriction enzymes were obtained from Invitrogen, except *PacI* and *AscI*, which were obtained from New England Biolabs (Hitchin, Hertfordshire, UK). The sequence mix was obtained from Perkin Elmer (Foster City, CA).

The pBinPLUS vectors containing the inserts were transformed to *Agrobacterium tumefaciens*, strain LBA4404 competent cells, using a freeze thaw protocol (Hofgen and Willmitzer, 1988). Colonies were checked after

transformation by back-transformation to *E. coli* XL1 supercompetent cells (Stratagene) and subsequent restriction enzyme digestion and sequencing. Basic DNA manipulations were carried out as previously described (Sambrook et al., 1989).

The three constructs were transformed to tobacco. For tobacco transformation, a standard leaf disc transformation and regeneration protocol was used essentially as described previously (Horsch et al., 1985). As control, leaf cuttings were also transformed with LBA4404 containing the original pBinPLUS vector. Furthermore, some non-transformed leaf cuttings were taken through the same regeneration process. Plants rooting on kanamycin (50 mg L⁻¹) containing Murashige and Skoog medium supplemented with 20 g L⁻¹ Suc were transferred to soil in the greenhouse.

RNA Analysis

Leaf tissue (young leaf, 2 cm long, not fully developed) was taken from young plants in the greenhouse and directly frozen in liquid N₂.

Total RNA was isolated from 200 mg of frozen tissue ground in liquid N₂, which was vigorously mixed (30 s) with a preheated mixture of 0.6 mL of acid phenol (80°C) and 0.6 mL of RNA extraction buffer containing 100 mM NaAc, pH 4.8, 100 mM LiCl, 10 mM EDTA, and 10% SDS. Another 0.6 mL of chloroform:isoamylalcohol (24:1) was added, and again the mixture was vigorously mixed for 30 s. The mixture was centrifuged at 9,400g for 15 min at 4°C. One-third volume of 8 M LiCl was added to the aqueous top phase, and the resulting mixture was incubated for 1 h at –80°C, subsequently thawed, and centrifuged at 12,000g and at 4°C for 15 min. The resulting pellet was washed with 70% ethanol, air dried, and dissolved in water.

Northern blotting was performed as described previously (Lücker et al., 2001). Probes used for the northern blots were the full-length original cDNA clones excised from the pBluescript multiple cloning site. Labeling, hybridization, stripping, and subsequent hybridization with a ribosomal cDNA as loading control were carried out as described previously (Lücker et al., 2001).

Crossings and Seed Analysis

Primary transformants were selfed and crossed. One hundred seeds of control or transgenic plants were plated on solid Murashige and Skoog medium supplemented with 10 g L⁻¹ Suc and 100 mg L⁻¹ kanamycin to select for transgenics. Plants giving high expression and containing one insertion of the different genes were crossed. First TER-6 was crossed with PIN-26 resulting in offspring containing and expressing both genes as selected by GC-MS analysis. Such a plant was crossed with LIM-21, and the resulting progeny contained a plant with all the three genes inserted in its genome. From this plant line, several cuttings were made. New plants were also regenerated in vitro from leaf cuttings, and regenerated plantlets were transferred to the greenhouse.

Volatile Analysis

To measure the release of volatiles by the tobacco plants, the headspace of leaves and flowers was analyzed on intact plants in the greenhouse as well as on detached leaves and flowers from the plants. For intact plants and seedlings, a glass funnel was used to enclose the plant part to be analyzed, and aluminum foil was used as a seal. A 100- μ m polydimethylsiloxane-coated SPME fiber (Supelco, Belfonte, PA) was used to capture the volatiles released from the plant tissue. Volatile sampling was carried out during 30 min and repeated several times. The sample was injected in a GC-MS by thermal desorption, as was published earlier (Lücker et al., 2001). This method was used to screen the progeny of the crossing experiments for the desired combination of terpenes.

To verify that all plants to be used as parents in the crossing experiment did produce the expected terpenes, nonquantitative analysis of the endogenous content of volatiles was performed using tissue ground in liquid N₂ and 5 M CaCl₂ solution and subsequent SPME sampling of the headspace of this extract as described previously (Lücker et al., 2001). The compounds were identified by comparison of GC retention times and mass spectra with those of authentic reference compounds.

Detached flowers or leaves were also used for headspace trapping onto Tenax TA (20/35 mesh; Alltech, Breda, the Netherlands). Four different flower developmental stages were used for volatile sampling (Goldberg,

1988; Koltunow et al., 1990): stage 8, corolla elongating, petals green and slightly open; stage 10, corolla limb beginning to open, petal tips pink; stage 11, corolla limb halfway open, stigma and anthers visible; and stage 12, flower open, anthers dehiscent, corolla limb fully expanded and deep pink.

During the 24-h sampling period, all stages developed to the next stage. Five flowers of each stage of the control and of the plant line with the three monoterpene synthases were taken for simultaneous analysis of volatile emission. The flowers or leaves were directly placed onto green floral foam blocks (Smithers-Oasis Belgium N.V., Houthalen, Belgium) saturated with water and wrapped in aluminum foil before they were enclosed in 1-L glass jars closed with a Teflon-lined lid equipped with inlet and outlet. A vacuum pump was used to draw air through the glass jar at approximately 100 mL min⁻¹, with the incoming air being purified through a glass cartridge (140 × 4 mm) containing 150 mg of Tenax TA. At the outlet, the volatiles emitted by the detached flowers or leaves were trapped on a similar Tenax cartridge. Volatiles were sampled during 24 h. Cartridges were eluted using three times 1 mL of redistilled pentane-diethyl ether (4:1). (+)-3-Carene (212 ng) was added to the eluent, as an internal standard. The samples were either used directly or after concentration under a stream of nitrogen. A volume of 2 µL was injected in a HP 5890 series II gas chromatograph equipped with an HP5-MS column (30-m × 0.25-mm i.d., 0.25-µm film thickness) and an HP 5972A Mass Selective Detector as described previously (Bouwmeester et al., 1999). GC oven temperature was programmed at an initial temperature of 45°C for 1 min with a ramp of 10°C min⁻¹ to 280°C and final time of 10 min.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining this permission will be the responsibility of the requestor.

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